Markers of instability in high-risk carotid plaques are reduced by statins

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Background: **Macrophage infiltration and expression of matrix metalloproteinase-9 (MMP-9) are markers of high-risk atherosclerotic carotid plaques and strong indicators of plaque instability. Use of statins is associated with a decreased risk of stroke and reportedly improves stability of atherosclerotic plaques, but available data addressing the mechanism of this effect are conflicting.**

Methods: **We retrospectively analyzed data from 94 consecutive patients with internal carotid artery stenosis who underwent carotid endarterectomy. Excised plaques underwent systematic quantitative immunohistochemical analysis to determine the percentage of macrophage area and the percentage of MMP-9 area. Associations between percentage of macrophage area and percentage of MMP-9 area and use of statins and cerebrovascular disease were examined by univariate and multivariate analysis.**

Results: **We found significantly higher values of percentage of macrophage area and of MMP-9 area in recently symptomatic (n = 26) compared with asymptomatic (n = 68) internal carotid artery stenoses: median (IQR) percentage of macrophage area was 2.29 (1.53-4.129) vs 0.53 (0.27-0.96) and percentage of MMP-9 area was 0.61 (0.36-0.89) vs 0.08 (0.02-0.27; both $P < .0005$). Patients treated with statins (n = 49) showed lower percentage values of macrophage area and MMP-9 area than untreated patients: the percentage of macrophage area was 0.54 (0.31-1.18) vs 1.03 (0.57-2.08; $P = .01$) and percentage of MMP-9 area was 0.06 (0.02-0.22) vs 0.36 (0.16-0.62; $P < .0005$). These associations between statin treatment and percentages of macrophage area and MMP-9 area did not change after controlling for symptomatic cerebrovascular disease and the effects of other potential confounders in multivariable analysis.**

Conclusions: **Our results confirm the value of percentage of macrophage area and percentage of MMP-9 area as markers of plaque instability and provide further evidence to support the hypothesis that statins reduce inflammatory responses and thereby stabilize carotid atherosclerotic plaques. (J Vasc Surg 2008;47:513-22.)**

Ischemic strokes are frequently caused by thromboemboli that arise from carotid atherosclerotic plaques.[1] Inflammatory processes in general, and monocyte-derived macrophages in particular, play a critical role in plaque progression and plaque destabilization.[2-4] Several studies have reported an increase in the number of macrophages in symptomatic internal carotid artery (ICA) plaques,[5-7] although in one study such a difference was not observed.[8]

Continued macrophage infiltration is believed to promote inflammatory signaling and contribute to enhanced proteolysis of the extracellular matrix by matrix metalloproteinases (MMPs). Matrix metalloproteinase-9 selectively degrades gelatin, collagen type IV and V, thereby weakening the protective fibrous cap that covers plaque lesions and making plaques more prone to rupture.[9] Higashikata et al[10] found that patients with symptomatic ICA stenoses had elevated levels of MMP-9, but not MMP-1 or MMP-3, in rupture-prone areas of plaque compared with stable areas, consistent with an important role for MMP-9 in plaque destabilization.

Several randomized and placebo-controlled trials have demonstrated that statins reduce strokes as well as transient ischemic attacks (TIAs) in patients with coronary artery disease (CAD), regardless of their initial cholesterol levels.[11-14] The Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial showed that after high-dose therapy with atorvastatin, the risk of stroke was reduced even in patients without active CAD.[15] These findings suggest that statin-related therapeutic effects involving cervical and cerebral arteries may be independent of cholesterol control, consistent with observations that statins mediate numerous pleiotropic effects, including anti-inflammatory effects that may influence plaque stability.[16-18]

It has been suggested that changes in the levels of macrophage infiltration and expression of MMP-9 can be used as markers of the anti-inflammatory effect of statin treatment on carotid plaques. Some studies have reported reduced MMP-9 levels with statin treatment,[19,20] and in a small but prospective cohort analysis, Crisby et al[21] observed reduced numbers of macrophages in symptomatic ICA stenoses after therapy with 40 mg of pravastatin. In the latter study, however, levels of MMP-9 expression were unaffected. Moreover, an analysis of carotid plaques from nearly 400 patients found that macrophage infiltration was not reduced but enhanced in patients with preoperative statin treatment and that MMP-9 expression was unaf-

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Competition of interest: none.

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fected.[22] Thus, the relationship between macrophage infiltration, MMP-9, and statin treatment in carotid plaques remains unclear.

In the present study, we used a systematic quantitative histochemical method applied to standardized immunohistochemically processed sections of carotid plaques to examine the relationship between macrophage infiltration and MMP-9 expression on the one hand, and statin treatment and the occurrence of symptomatic CVD on the other. We also evaluated sections for morphologic features of indirect plaque instability, such as plaque rupture, large lipid core, surface thrombus, and intraplaque hemorrhage, and correlated them with the immunohistochemical staining results.

METHODS

Patients. We screened 101 consecutive patients with high-grade ICA stenosis and planned carotid endarterectomy (CEA). Seven patients with nonatherosclerotic stenosis (eg, radiation-induced stenosis, restenosis) were excluded, leaving 94 patients. The study took place during a period of 27 months. Each patient's cardiovascular risk factors and medication history (drug types, dosage, duration) were documented. Fasting blood samples were collected on the day of surgery. Study patients were classified as having either asymptomatic or symptomatic ICA stenosis according to the North American Symptomatic Endarterectomy Trial criteria (NASCET).[23] Approval for this study was obtained from the institutional ethics committee. Informed consent was obtained from all patients before enrollment in the study.

Tissue preparation, immunohistochemistry, and histopathology. After surgery, ICA plaque specimens were immediately rinsed in 0.9% sodium chloride solution before fixation in a 4.5% (w/v) buffered formalin solution for 24 hours. Specimens were subsequently decalcified using ethylenediaminetetraacetate (EDTA) solution (0.5 mol/L) for 24 hours. Thereafter, specimens were cut in 3-mm-thick consecutive transverse sections, each cut beginning from the proximal end of the lesion. All sections were cut, consecutively, from the same plaque and were ordered, as indicated, from the proximate to distal end of the lesion, and sections were from the full extent and length of the surgically excised lesion using similar methods to those published by others.[24] Numbered sections were embedded side-by-side in paraffin using standard procedures. Altogether, an average of five transverse sections per patient were processed.

From these paraffin blocks, thin (4-μm) sections were obtained for immunohistochemical detection of CD68 (KP-1, 1:75 dilution Dako, Glostrup, Denmark) and MMP-9 (15W2, 1:25 dilution; Novocastra, Newcastle upon Tyne, United Kingdom). Detection of CD68 was done according to the manufacturer's instructions using a streptavidin-biotin system (LSAB kit, Dako). The immunohistochemical staining for detection of MMP-9 was done according to the manufacturer's instructions using the universal immunoenzyme polymer method (Histofine Simple Stain AP MULTI, Nichirei, Tokyo, Japan). For both stainings, alkaline phosphatase was used as the reporter enzyme and Fast Red chromogen (Sigma-Aldrich, St Louis, Mo) was used as the staining agent. For both stainings, color development was examined under a light microscope and stopped by immersing the slides in Tris buffer. Nuclear counterstaining was obtained using hematoxylin and the slides were mounted with aqueous mounting medium. All specimens were stained using the same procedures. Tonsil biopsy specimens were used as a positive control for CD68, and liver biopsy specimens were used as a positive control for MMP-9.

Staining and processing of all slides was accomplished during three consecutive days to control for staining variable (eg, quality and condition of solutions, materials, and room temperature) such that there is little or no variability in the staining conditions. So, for example for CD68 staining, up to 40 slides where stained in one session.

Additional transverse sections from paraffin blocks were taken and stained with elastin van Gieson and hematoxylin and eosin.

Immunohistochemical stainings were evaluated and quantified by two independent observers (H. K., N. A.) and an independent histopathologist (W. W.) who were blinded to the patient's medical or drug history. Analysis of morphologic features of indirect plaque instability was done by H. K., and the results were analyzed by an independent pathologist (W. W.). Morphologic features were graded as defined by Redgrave et al.[24] Cap rupture was recorded if there was clear communication between lipid core and lumen with a break between in the fibrous cap. Large lipid core was defined as amorphous material containing cholesterol crystals that occupied >50% of thickness of plaque or >25% of total cross section area. In addition, a giant lipid core was defined when definition of large lipid core was fulfilled in three cross sections or ≥50% of all cross sections and at least one lipid core occupied >75% of total cross-section area. Intraplaque hemorrhage was recorded if there was an area of erythrocytes within the plaque causing disruption of plaque architecture. Surface thrombus was defined as organized collection of fibrin and red blood cells in the vessel lumen.

Images were acquired using a motorized Axioplan 2 imaging microscope system and an Axiocam Hrc digital camera in conjunction with Axiovision 4.2 software at ×50 optical magnification. High-resolution images were merged from multiple overlapping images using a motorized microscope stage and the "stitching" technique of the software module Panorama. A mean number of 43 single images per section were joined (see Fig 1). Semiautomatic measurements were made using the software module AutoMeasure (all hardware and software products were from Carl Zeiss, Göttingen, Germany). All images were taken with the same standardized settings, including exposure time, filter settings, brightness, and contrast.

A color threshold mask was defined for the area of macrophage infiltration, the area of MMP-9 staining, as well as the total section area, to compute the percentage of red-colored immunostaining in relation to the total section

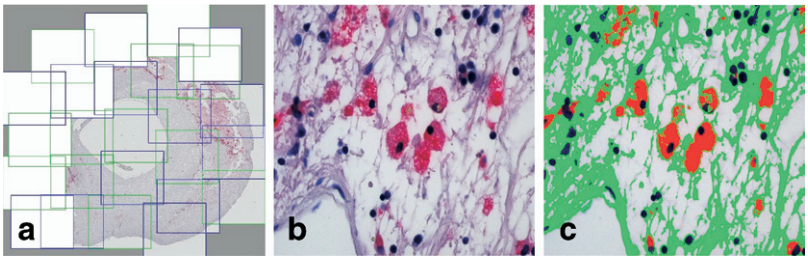

Fig 1. Quantification of the content of macrophages and matrix metalloproteinase-9 within atherosclerotic carotid plaques. **Panel a,** High-resolution image during processing. Single, overlapping images, framed in blue and green, were joined using the Panorama software stitching technique. As a result, we were able to zoom in on the details and quantitatively analyze the image (×50 original magnification). **Panel b,** Representative cross-section of macrophage immunoreactivity is shown at high resolution (×400 original magnification). **Panel c,** Representation of Fig 2, *b* during the process of specific signal detection at ×400 original magnification. A color threshold mask was defined for the total section areas as well as the areas of macrophage infiltration to detect the percentage of red-colored immunostaining areas compared with the total section area (see Evaluation of Immunohistochemical Stainings). We determined the size of the area occupied by CD68-positive cells planimetrically and calculated the total percentage of macrophage-rich areas in relation to the combined surface area measurements of all cross sections per specimen (sum of macrophage-rich areas of all cross sections in $\mu m^2 \times 100$/sum of surface area of all cross sections in μm^2). The total percentage of MMP-9 positive areas was determined by a corresponding approach.

area. This mask was set to detect even small stained areas. Macrophages were present in dense, often confluent, infiltrates, making the delineation of individual cells impossible. Cell nuclei surrounded by red staining were considered to be a part of macrophage-positive areas. This analysis did not distinguish between a concentrated accumulation or scattered distribution of macrophages and MMP-9. The same threshold was applied to all stainings.

We determined the size of the area occupied by CD68-positive cells planimetrically and calculated the total percentage of macrophage-rich areas in relation to the combined surface area measurements of all cross sections per specimen (PMA-sum of macrophage-rich areas of all cross sections in $\mu m^2 \times 100$/sum of surface area of all cross sections in μm^2). The percentage of MMP-9 area (PMMP-9A) was calculated in the same manner (Fig 1).

Statistical analysis. Categoric data are presented as numbers and percentages (%), and continuous numeric data are presented as median and interquartile range (IQR) because all numeric variables were not normally distributed. Unadjusted associations between statin treatment and other baseline variables and between baseline variables and PMA/PMMP-9A were analyzed using the χ^2 test or the Fisher exact test where appropriate for categoric variables and the Mann-Whitney *U* test for continuous numeric variables. Associations between statin treatment and PMA/PMMP-9A adjusted for CVD and other baseline variables for which a significant effect was found in the univariate analysis ($P < .2$ for inclusion) were analyzed by multiple logistic regression analysis (stepwise backward, $P < .1$ for exclusion) using PMA/PMMP-9A as binary outcomes (above or below the median), because the assumptions for multiple linear regression were not met. Significance of associations was calculated with the likelihood ration test for heterogeneity. A value of $P < .05$ was considered statistically significant.

To study the reproducibility of the assessments of PMA and PMMP-9A, two independent observers evaluated all stainings. Interobserver agreement was assessed by calculating the intraclass correlation coefficient (ICC). Because interobserver agreement was equivalent for both PMA and PMMP-9A (ICC >97%), the mean values of the two independent measurements for each case were used for statistical analysis. Statistical analyses were performed with the SPSS 12.0 (SPSS Inc, Chicago, Ill) and Stata 9.2 (StataCorp, College Station, Tex) software packages.

RESULTS

Patient characteristics. Patient data are summarized in Table I. According to the NASCET criteria, 26 of 94 patients (28%) were characterized as symptomatic and 68 (72%) as asymptomatic. The median time between the ischemic event and CEA was 17.5 days (IQR, 10-28 days).

At the time of enrollment in the study, 49 patients (52% of all patients) were being treated with statins. All were taking lipophilic statins, including atorvastatin in 34 (69%), simvastatin in 14 (29%), and fluvastatin in one (2%). The median duration of therapy was 12 months overall, 7 months for atorvastatin, and 24 months for simvastatin. The patient on fluvastatin had been taking the medication for 12 months. Statins were prescribed to 37 of 68 patients (54%) with asymptomatic ICA stenosis and 12 of 26 (46%) with symptomatic ICA stenosis ($P = .5$). Eight patients had become symptomatic during statin therapy. Four patients received statins after an ischemic event but before CEA; the median duration of therapy was 34.5 days.

Of the variables listed in Table I, only the value for apolipoprotein B (Apo B) was significantly different between symptomatic and asymptomatic patients, with lower values in patients with symptomatic ICA stenosis. The grade of stenosis as well as the use of other medications (nonsteroidal anti-inflammatory drugs, immunosuppres-

Table I. Baseline characteristics of patients from statin and no statin groups

Variables[a]	*Statin (n = 49)*	*No statin (n = 45)*	P
Symptomatic ICA stenosis	12 (25)	14 (31)	.47[b]
Grade of ICA stenosis, %	90 (80-90)	85 (80-90)	.07[b]
Age, years	68 (58.5-75.5)	64 (59.5-71.5)	.36[b]
Males	31 (63)	35 (78)	.12[b]
Coronary artery disease	25 (51)	25 (56)	.66[b]
Myocardial infarction	10 (20)	8 (18)	.75[b]
Peripheral vascular disease	15 (31)	16 (36)	.61[b]
Hypertension	49 (100)	40 (89)	.02[b]
Hyperlipidemia	49 (100)	19 (42)	<.0005[b]
Diabetes mellitus	16 (33)	16 (36)	.77[b]
Body mass index, kg/m^2	26.8 (24.4-28.7)	25.6 (23.7-28.6)	.41[c]
Smoking habits, pack years	0 (0-35)	0 (0-41.5)	.46[c]
Ex-smokers	9 (18)	10 (22)	.64[b]
Alcohol abuse	3 (6)	6 (13)	.24[b]
Family history of vascular diseases[d]	27 (55)	20 (44)	.30[b]
Serology			
Cholesterol, mg/dL	157 (133.5-181.5)	192 (172.5-215)	<.0005[c]
Triglycerides, mg/dL	180 (123-224.5)	177 (119-254.5)	.69[c]
LDL, mg/dL	78 (54.5-95.5)	118 (92-133)	<.0005[c]
HDL, mg/dL	45 (38-49)	43 (37.5-48.5)	.69[c]
C-reactive protein, mg/dL	0.3 (0.1-0.8)	0.2 (0.1-0.45)	.55[c]
Apolipoprotein A1, mg/dL	123 (106.5-136)	122 (109-133.5)	.87[c]
Apolipoprotein B, mg/dL	78 (65-90)	95 (86-116.5)	<.0005[c]
Lipoprotein (a), mg/dL	11.6 (4.9-51.4)	22.3 (6.8-43.7)	.25[c]

LDL, Low-density lipoprotein; *HDL*, high-density lipoprotein.
[a]Categoric variables are presented as number (%), continuous variables median (interquartile range).
[b]By χ^2 test, $P < .05$ was significant.
[c]Mann-Whitney *U* test; $P < .05$ was significant.
[d]Cerebrovascular, cardiac, or peripheral vascular disease among first and second degree relatives.

sants, antibiotics, angiotensin-converting enzyme inhibitors, calcium antagonists, angiotensin receptor inhibitors, β-blockers, nitrates, diuretics, insulin, oral antidiabetics) was not significantly different between patients with asymptomatic and symptomatic ICA stenosis or between patients with or without statin therapy (data not all shown). In the statin group, significantly higher proportions of patients had hyperlipidemia and hypertension. Statin-treated patients had lower median concentrations of cholesterol, low-density lipoprotein (LDL), and Apo B, consistent with successful treatment of hyperlipidemia, but did not differ from untreated patients in other blood test results or risk factors (Table I). Notably, all patients had normal levels of C-reactive protein, indicating that no confounding acute inflammatory disease was present.

Semiquantitative immunohistochemistry and histopathology. We first validated our immunohistochemical analysis for detection of macrophages and MMP-9. Fig 2 shows representative sections that were stained with antibodies for macrophages and MMP-9, which were readily detected by red staining. Appropriate staining of macrophages and MMP-9 was confirmed using tonsil and liver biopsy specimens, respectively, as positive controls. Overall, the median PMA was 0.86 (IQR, 0.42-1.53) and the median PMMP-9A was 0.16 (IQR, 0.05-0.46).

Significantly lower values of PMA and PMMP-9A were found in asymptomatic compared with symptomatic ICA stenosis (both $P < .0005$; Fig 3). The difference between asymptomatic and symptomatic patients seemed to be more pronounced for MMP-9 staining compared with macrophage infiltration. Among 26 patients with symptomatic stenosis, there was weak evidence for a correlation between time from symptoms in days and PMA (Spearman ρ, −0.38; $P = .06$), with higher PMA values in patients with more recent symptoms. There was no evidence for a correlation between time from symptoms and PMMP-9A (Spearman ρ, −0.03; $P = .09$). Five of the group of patients with asymptomatic ICA stenosis had a history of carotid-territory ischemic events older than 120 days related to their ICA stenosis. Values of PMA and PMMP-9A were lower in these five patients than in patients with a symptomatic ICA stenosis.

The assessment of associations between morphologic features of indirect plaque instability (Fig 4) and symptomatic stenosis and the relationships between those features and PMA and PMMP-9A are summarized in Tables II and III. Plaque rupture, large and giant lipid core, and plaque hemorrhage were all associated with symptomatic stenosis and with higher values of PMA. Significantly higher PMMP-9A values were found in ruptured plaques and in plaques with giant cores.

Associations between statins and inflammatory markers. Median PMA and PMMP-9A values were twofold and sixfold lower, respectively, in the 49 patients receiving statins before CEA than in the 45 untreated

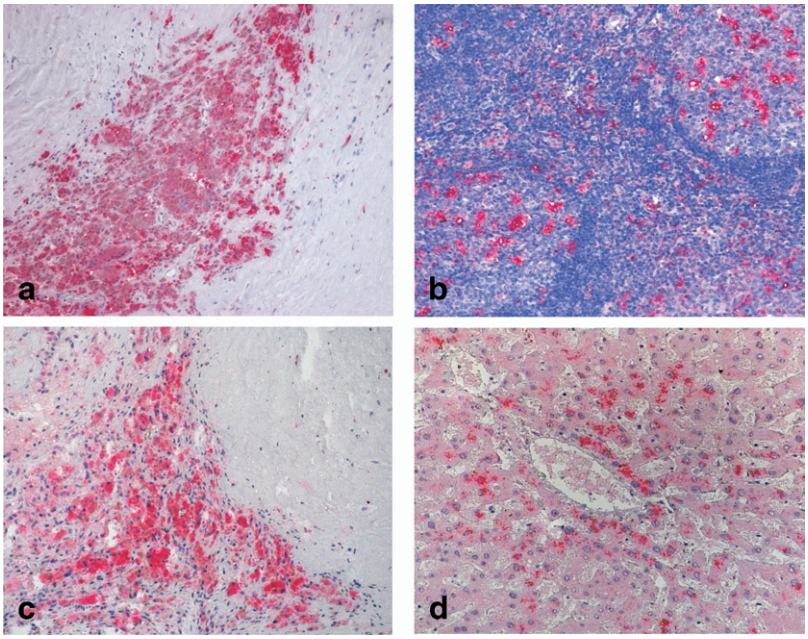

Fig 2. Validation of immunohistochemical analysis for detection of macrophages and matrix metalloproteinase-9 (MMP-9). **Panel a,** Representative cross section shows specific immunoreactivity for macrophages. Anti-CD68 staining results in a granular cytoplasmic staining with red dye precipitates (×100 original magnification). **Panel b,** Positive control shows specific immunoreactivity for macrophages in a tonsil biopsy specimen. **Panel c,** Representative cross section shows specific immunoreactivity for MMP-9. The color pattern resulting from anti-MMP-9 staining is cytoplasmic (×100 original magnification) **Panel d,** Positive control shows specific immunoreactivity for MMP-9 in a liver biopsy specimen.

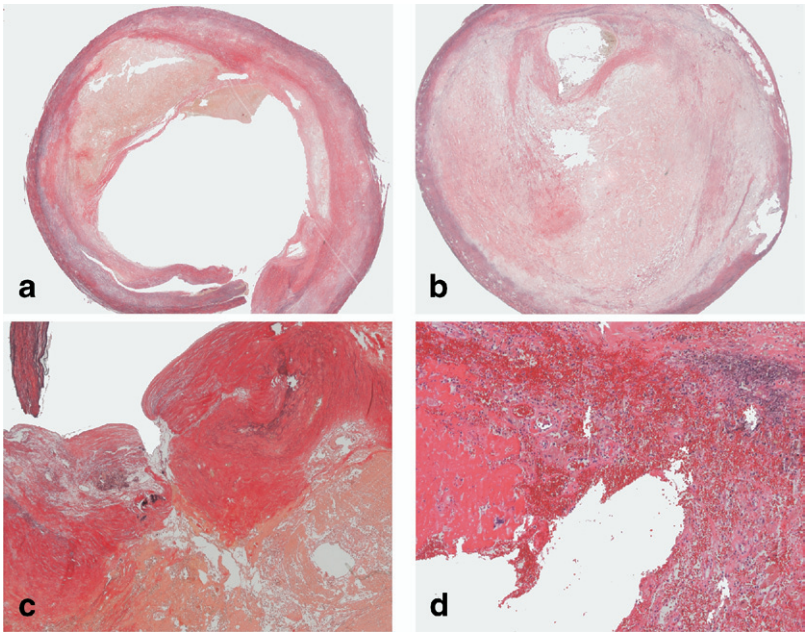

Fig 3. Characteristic morphologic features of indirect plaque instability. **Panel a,** Representative cross section showing plaque with thin fibrotic cap and a fixed thrombus in the lumen of the vessel (staining with elastin van Gieson, ×15 original magnification). **Panel b,** Plaque cross-section with a very large lipid core (staining with elastin van Gieson, ×15 original magnification). **Panel c,** Plaque cross-section shows a plaque rupture with plaque content erupting from the lipid core (staining with elastin van Gieson, ×50 original magnification). **Panel d,** Plaque cross-section shows an intraplaque hemorrhage (staining with hematoxylin and eosin, ×50 original magnification).

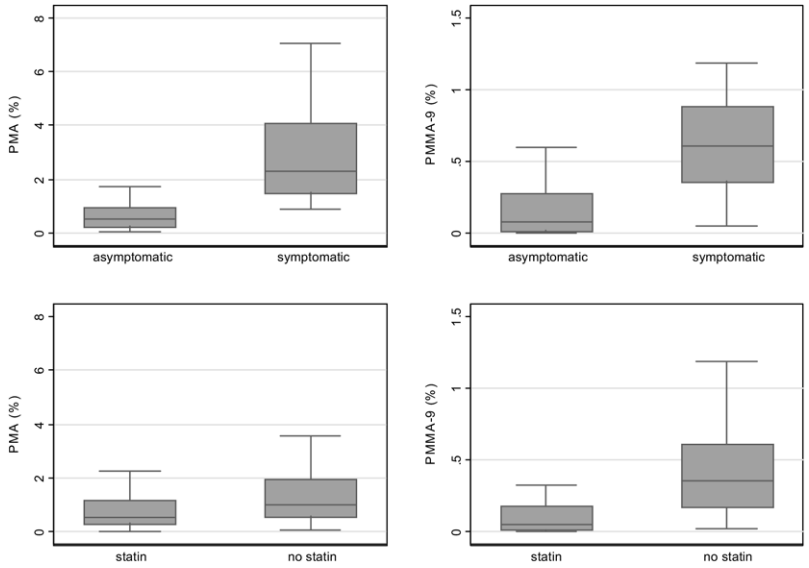

Fig 4. Box and whisker plots show the distribution of percentage of macrophage-rich area *(PMA)* in relation to the surface area of all cross-sections per specimen and the total percentage of matrix metalloproteinase-9-positive areas *(PMMA-9)* in relation to the surface area of all cross-sections per specimen in different patient groups. Boxes represent the interquartile range (IQR) and the centerlines represent the median. The whiskers extend to the upper and lower adjacent values ($\pm 1.5 \times$ IQR).

Table II. Unadjusted associations between baseline characteristics and plaque morphology, and percentage of macrophage-rich area and percentage of matrix metalloproteinase-9 area

Variable	*PMA >0.86%, OR (95% CI)*	P[a]	*PMMP-9A >0.16%, OR (95% CI)*	P[a]
Baseline characteristics				
Statin therapy	0.4 (0.17-0.88)	.02	0.1 (0.05-0.3)	<.0001
Symptomatic stenosis	>1000	<.0001	14.1	<.0001
Age >66 years	0.5 (0.20-1.05)	.06	0.7 (0.29-1.47)	.3
Grade of stenosis>85%	1.3 (0.57-3.04)	.5	1.3 (0.57-3.04)	.5
Male sex	1.0 (0.41-2.42)	1.0	1.2 (0.51-3.0)	.7
Coronary artery disease	0.6 (0.26-1.35)	.2	1.2 (0.52-2.67)	.7
Myocardial infarction	1.0 (0.36-2.79)	1.0	0.8 (0.27-2.13)	.6
Peripheral vascular disease	0.7 (0.31-1.77)	.5	1.1 (0.47-2.60)	.8
Hypertension	0.6 (0.10-4.09)	.6	0.6 (0.10-4.09)	.6
Hyperlipidemia	1.2 (0.50-3.06)	.6	0.5 (0.21-1.32)	.2
Diabetes mellitus	0.7 (0.29-1.61)	.4	1.2 (0.51-2.84)	.7
BMI (per unit increase)	1.0 (0.90-1.12)	1.0	1.0 (0.94-1.16)	.4
Current smoker	1.6 (0.68-3.55)	.3	1.6 (0.68-3.55)	.3
Ex-smoker	0.67 (0.24-1.86)	.4	0.88 (0.31-2.40)	.8
Alcohol abuse	1.3 (0.32-5.10)	.7	00.78 (0.20-3.11)	.7
Family history of vascular diseases	1.1 (0.48-2.44)	.8	0.8 (0.34-1.74)	.5
Plaque morphology				
Plaque rupture	15.8 (1.96-127.10)	.0004	15.8 (1.96-127.10)	.0004
Large core	4.6 (1.37-15.14)	.007	2.3 (0.80-6.89)	.1
Giant core	3.3 (1.27-8.62)	.01	3.3 (1.27-8.62)	.01
Plaque hemorrhage	2.8 (1.05-7.26)	.03	0.9 (0.36-2.24)	.8
Thrombus	5.3 (1.08-26.18)	.02	1.9 (0.51-6.91)	.3

BMI, Body mass index; *CI,* confidence interval; *OR,* odds ratio; *PMA,* percentage of microphage-rich areas; *PMMP-9A,* percentage of matrix metalloproteinase-9 area.

[a]Likelihood ratio test of heterogeneity. Values of $P < .05$ are significant.

patients ($P = .01$ and $P < .0005$, respectively; Fig 4). When the effects of individual statins were analyzed separately, simvastatin was associated with lower PMA ($P < .003$) and PMMP-9A values ($P < .0009$), whereas atorvastatin-treated patients had lower values for PMMP-9A ($P < .00001$) but not for PMA ($P = .1$).

Because assumptions for multiple linear regression analysis were not met for the relationship between statin

Table III. Associations between symptomatic stenosis, plaque morphology, and inflammatory markers

	No.	*Symptomatic (n = 26), No. (%)*	*Asymptomatic (n = 68), No. (%)*	P[a]	*PMA Median (IQR)*	P[b]	*PMMP-9A Median (IQR)*	P[b]
Plaque rupture	13	9 (35)	4 (6)	<.0001	2.32 (1.59-3.57)	.0002	0.36 (0.33-0.63)	.005
No plaque rupture	81	17 (66)	64 (94)		0.70 (0.33-1.22)		0.12 (0.03-0.45)	
Large core	76	25 (96)	51 (75)	.02	0.98 (0.50-1.71)	.0001	0.22 (0.05-0.49)	.1
No large core	18	1 (4)	17 (25)		0.29 (0.09-0.53)		0.07 (0.24-0.28)	
Giant core	27	13 (50)	14 (21)	.005	1.34 (0.74-1.93)	.001	0.44 (0.08-0.63)	.006
No giant core	67	13 (50)	54 (79)		0.54 (0.29-1.21)		0.11 (0.03-0.35)	
Plaque hemorrhage	25	12 (46)	13 (19)	.0008	1.20 (0.61-2.27)	.01	0.14 (0.06-0.69)	.24
No hemorrhage	69	14 (54)	55 (81)		0.70 (0.31-1.33)		0.18 (0.03-0.44)	
Thrombus	11	4 (15)	7 (10)	.49	1.07 (0.88-2.72)	.08	0.29 (0.01-0.35)	.86
No thrombus	83	22 (85)	61 (90)		0.74 (0.33-1.52)		0.15 (0.05-0.46)	

IQR, Interquartile range; *PMA,* percentage of microphage-rich areas; *PMMP-9A,* percentage of matrix metalloproteinase-9 area.
[a]Mann-Whitney *U* test.
[b]χ^2 test.

treatment and PMA and PMMP-9A, binary outcome variables that categorized patients as above (1) or below (0) the median PMA and PMMP-9A values, respectively, were used for further analysis. Table II summarizes the results of the unadjusted analysis of associations between baseline variables and PMA/PMMP-9A. The serologic parameters listed in Table I were not significantly associated with PMA or PMMP-9A in univariable analysis; there was also no association between all medications other than statins and PMA or PMMP-9A.

Statin therapy, symptomatic stenosis, age older than median age of 66 years, coronary artery disease, and hyperlipidemia were associated with PMA or PMMP-9A at the $P < .2$ level and were therefore included in the multivariable models. In the final models, only statin therapy and symptomatic stenosis were independently associated with PMA, with odds ratios (OR) of 0.3 (95% confidence interval [CI], 0.1-0.84) and >100 (95% CI not measurable), respectively, and PMMP-9A, with ORs of 0.1 (95% CI, 0.02-0.2) and 29.7 (95% CI, 6.11-144.53), respectively. As a last step, all baseline variables, including vascular risk factors, serologic parameters, and medications, were added again, individually to the model, but none of them influenced the adjusted effects of statins and symptomatic stenosis, nor did any of them become significant in the adjusted analysis at this stage. Thus, the effects of statin therapy and symptomatic stenosis on PMA and PMMP-9A were independent of each other and of all other variables.

When associations between morphologic features of indirect plaque instability and PMA/PMMP-9A were assessed using binary outcomes, all features were significantly associated with PMA values above the median, and plaque rupture and giant plaque core were associated with PMMP-9A values above the median. These associations remained significant after adjusting for statin therapy using standard logistic regression analysis.

DISCUSSION

There are three important findings from the present study. Our data indicate that:

1. PMA and PMMP-9A are suitable markers of plaque instability, with significantly higher values of PMA and PMMP-9A present in symptomatic compared with asymptomatic ICA plaques;
2. higher values of PMA and PMMP-9A are associated with plaque rupture and giant lipid core in carotid stenosis; and
3. treatment with therapeutic doses of statins is associated with reduced values of PMA and PMMP-9A in ICA plaques.

The first two of these findings confirm the generally accepted concept of a critical association between inflammatory markers and instability of atherosclerotic carotid plaques.[7,25] However, the newly defined marker "giant lipid core" seems to be a better a marker of indirect plaque instability than previous markers such as large lipid core, especially in combination with other markers. In earlier studies, a large lipid core[24] was found in >80% of all plaque specimens. A giant lipid core, on the other hand, was absent in 79% of asymptomatic carotid stenosis in our study. It thus seems that giant lipid core is a more sensitive and specific inflammatory marker; therefore, using multiple markers of plaque instability may be the most sensitive methodology.

The third finding provides important new evidence on the possible mechanism involved in the beneficial effects of statins. Previously, important questions involved the known dissociation between cholesterol levels and clinical benefit of statins with regard to cerebrovascular risk and the uncertain effect of statins on inflammatory markers in atherosclerotic plaques.[21,22]

Previous work has shown a clear association between inflammatory markers, plaque instability, and risk of stroke and TIAs, whereas other studies have shown a clear association between use of statins and reduced risk of stroke.[21,26] On the grounds of these observations, it has been suggested that use of statins might be associated with a reduction in inflammatory markers, but this association had not previously been established, with available data being con-

flicting or contradictory.[19-22] Here, we obtained evidence that use of statins is associated with a reduction in inflammatory markers PMA and PMMP-9A, independently of cholesterol levels, providing important evidence supporting involvement of an inflammatory mechanism in the beneficial effects of statins.

Analyzing effects of individual statins separately, simvastatin was associated with lower PMA and PMMP-9A values. This effect was not found in atorvastatin-treated patients for PMA even though these patients showed lower values for PMMP-9A. This difference might be due to a type II error because the number of patients taking atorvastatin was very low. Also, the duration of therapy needed for the effect between different statins might be different. In this context, however, it must be mentioned that Verhoeven et al[22] found a significant increase of CD68-positive cells in carotid plaques obtained from statin-treated patients that was mainly attributable to atorvastatin-treated patients. In patients treated with atorvastatin, the increased amount of CD68-positive cells was not associated with increased protease activity. On the contrary, the authors showed a dose-dependent decrease in MMP activity in the atorvastatin group.

Statin-mediated inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase not only blocks synthesis of cholesterol but also affects synthesis of isoprenoids. These intermediates serve as lipid attachments for a variety of intracellular signaling molecules. Reduction of isoprenoids probably accounts for most of the pleiotropic effects observed with statin treatment.[16-18] The reduced number of macrophages in plaques of statin-treated patients could potentially be due to downregulation of monocyte adhesion molecules,[27] monocyte chemoattractant protein-1,[28] or suppression of oxidized LDL-induced macrophage proliferation.[29]

The attenuation of MMP-9 expression that we observed in the plaques of statin-treated patients might have been due to reduced macrophage content and diminished macrophage activation. However, the magnitude of the reduction in PMA was less than that of MMP-9 with statin therapy, suggesting that MMP-9 expression may serve as a better marker for plaque instability.

The nature of our study did not allow for conclusive statements regarding the activity of macrophages and of MMP-9. Gelatin zymography and immunoblotting was not done because we did not want to destroy the morphologic structure of the plaque to get material for this analysis. Further, we examined morphologic features of indirect plaque instability to look at plaque rupture, large lipid core, surface thrombus, and intraplaque hemorrhage and correlated them with the immunohistochemical staining results. In addition to MMP-9 and macrophage content, several other markers that may also contribute to plaque instability have been identified.[30-33]

We believe that the systematic, quantitative approach used here was critical for obtaining unambiguous data to address this important issue. We used sophisticated image detection and analysis to quantify macrophage infiltration and MMP-9 expression, which we were then able to correlate with clinical markers and use of statins. In our study the entire plaque was systematically sectioned and evaluated, with each plaque yielding >200 single, high-resolution images per patient. Immunohistochemical staining for macrophages and MMP-9 was carefully standardized for reproducibility, and high-resolution images were quantitatively analyzed, resulting in a detailed and comprehensive analysis of the entire plaque lesion.

Among the patients studied here, the statin group showed a significantly higher proportion with hyperlipidemia and hypertension. On the one hand, the median concentrations of cholesterol, LDL, and Apo B were lower than in the no statin group (Table I). This may indicate that compliance was high in the statin group, but it also emphasizes the importance of the statin effect, in view of the additive risk associated with hyperlipidemia and hypertension.

An important limitation of the present study was the length of time between symptom development and immunohistochemical assessment of the plaque. The median time between the qualifying event for a symptomatic ICA stenosis and surgery was 17.5 days. Ideally, the shortest possible time interval would be found between the ischemic event and the time when the plaque material is obtained. The relatively long time in our study raises the possibility that healing may have already started within the lesion, which might have diminished the magnitude of the effect that we observed.

Patients who had an ischemic event in the same carotid territory >120 days before surgery showed values of PMA and PMMP-9A similar to those of asymptomatic patients who never had an ischemic event, suggesting that a symptomatic ICA stenosis can lose its high-risk character. This, and our finding that PMA decreased over days from symptoms, is in line with the observation that the benefit of CEA declines after the first few weeks after acute symptoms.[34]

Another important limitation of the present study was the observational study design. A randomized, placebo-controlled design was considered not to be ethically justifiable due to the ever-growing evidence that statins exert significant plaque-stabilizing effects and that precisely those patients with a high degree of cerebrovascular risk are likely to benefit most from statin treatment.[35-37] In addition, the beneficial effects of early CEA in patients with symptomatic ICA stenosis have been clearly demonstrated,[34] thus precluding a study design with delayed intervention intended to evaluate the effect of preoperative use of a medication.

CONCLUSION

Our data provide evidence that use of statins attenuates the inflammatory response in carotid atherosclerotic plaques, thereby reaffirming the hypothesis, that the beneficial effects of statins on cerebrovascular events is at least mediated by the anti-inflammatory effects of statins on atherosclerotic plaques. Thus, the data suggest an essen-

tially mechanistic link between inflammation, plaque stability, and statin use.

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AUTHOR CONTRIBUTIONS

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Statistical analysis: HK, MB
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REFERENCES

1. Sacco RL, Ellenberg JH, Mohr JP, Tatemichi TK, Hier DB, Price TR, et al. Infarcts of undetermined cause: the NINCDS Stroke Data Bank. Ann Neurol 1989;25:382-90.
2. Golledge J, Greenhalgh RM, Davies AH. The symptomatic carotid plaque. Stroke 2000;31:774-81.
3. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105:1135-43.
4. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999; 340:115-26.
5. Bassiouny HS, Sakaguchi Y, Mikucki SA, McKinsey JF, Piano G, Gewertz BL, et al. Juxtalumenal location of plaque necrosis and neoformation in symptomatic carotid stenosis. J Vasc Surg 1997;26: 585-94.
6. Carr SC, Farb A, Pearce WH, Virmani R, Yao JS. Activated inflammatory cells are associated with plaque rupture in carotid artery stenosis. Surgery 1997;122:757-63.
7. Jander S, Sitzer M, Schumann R, Schroeter M, Siebler M, Steinmetz H, et al. Inflammation in high-grade carotid stenosis: a possible role for macrophages and T cells in plaque destabilization. Stroke 1998;29: 1625-30.
8. Lehtonen-Smeds EM, Mayranpaa M, Lindsberg PJ, Soinne L, Saimanen E, Jarvinen AA, et al. Carotid plaque mast cells associate with atherogenic serum lipids, high grade carotid stenosis and symptomatic carotid artery disease. Results from the Helsinki carotid endarterectomy study. Cerebrovasc Dis 2005;19:291-301.
9. Fatar M, Stroick M, Griebe M, Hennerici M. Matrix metalloproteinases in cerebrovascular diseases. Cerebrovasc Dis 2005;20:141-51.
10. Higashikata T, Yamagishi M, Higashi T, Nagata I, Iihara K, Miyamoto S, et al. Altered expression balance of matrix metalloproteinases and their inhibitors in human carotid plaque disruption: Results of quantitative tissue analysis using real-time RT-PCR method. Atherosclerosis 2006;185:165-72.
11. Plehn JF, Davis BR, Sacks FM, Rouleau JL, Pfeffer MA, Bernstein V, et al. Reduction of stroke incidence after myocardial infarction with pravastatin: the Cholesterol and Recurrent Events (CARE) study. The Care Investigators. Circulation 1999;99:216-23.
12. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. N Engl J Med 1998;339:1349-57.
13. Waters DD, Schwartz GG, Olsson AG, Zeiher A, Oliver MF, Ganz P, et al. Effects of atorvastatin on stroke in patients with unstable angina or non-Q-wave myocardial infarction: a Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) substudy. Circulation 2002;106:1690-5.
14. Collins R, Armitage J, Parish S, Sleight P, Peto R. Effects of cholesterol-lowering with simvastatin on stroke and other major vascular events in 20536 people with cerebrovascular disease or other high-risk conditions. Lancet 2004;363:757-67.
15. Amarenco P, Bogousslavsky J, Callahan A 3rd, Goldstein LB, Hennerici M, Rudolph AE, et al. High-dose atorvastatin after stroke or transient ischemic attack. N Engl J Med 2006;355:549-59.
16. Endres M. Statins and stroke. J Cereb Blood Flow Metab 2005;25: 1093-110.
17. Kwak BR, Mulhaupt F, Mach F. Atherosclerosis: anti-inflammatory and immunomodulatory activities of statins. Autoimmun Rev 2003;2: 332-8.
18. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. Arterioscler Thromb Vasc Biol 2001; 21:1712-9.
19. Aikawa M, Rabkin E, Sugiyama S, Voglic SJ, Fukumoto Y, Furukawa Y, et al. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. Circulation 2001;103:276-83.
20. Bellosta S, Via D, Canavesi M, Pfister P, Fumagalli R, Paoletti R, et al. HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages. Arterioscler Thromb Vasc Biol 1998;18:1671-8.
21. Crisby M, Nordin-Fredriksson G, Shah PK, Yano J, Zhu J, Nilsson J. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: implications for plaque stabilization. Circulation 2001; 103:926-33.
22. Verhoeven BA, Moll FL, Koekkoek JA, van der Wal AC, de Kleijn DP, de Vries JP, et al. Statin treatment is not associated with consistent alterations in inflammatory status of carotid atherosclerotic plaques: a retrospective study in 378 patients undergoing carotid endarterectomy. Stroke 2006;37:2054-60.
23. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. N Engl J Med 1991;325:445-53.
24. Redgrave JN, Lovett JK, Gallagher PJ, Rothwell PM. Histological assessment of 526 symptomatic carotid plaques in relation to the nature and timing of ischemic symptoms: the Oxford plaque study. Circulation 2006;113:2320-8.
25. Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PR, et al. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. Stroke 2000;31:40-7.
26. Aikawa M, Rabkin E, Sugiyama S, Voglic SJ, Fukumoto Y, Furukawa Y, et al. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. Circulation 2001;103:276-83.
27. Serrano CV Jr, Yoshida VM, Venturinelli ML, D'Amico E, Monteiro HP, Ramires JA, et al. Effect of simvastatin on monocyte adhesion molecule expression in patients with hypercholesterolemia. Atherosclerosis 2001;157:505-12.
28. Bustos C, Hernandez-Presa MA, Ortego M, Tunon J, Ortega L, Perez F, et al. HMG-CoA reductase inhibition by atorvastatin reduces neointimal inflammation in a rabbit model of atherosclerosis. J Am Coll Cardiol 1998;32:2057-64.
29. Senokuchi T, Matsumura T, Sakai M, Yano M, Taguchi T, Matsuo T, et al. Statins suppress oxidized low density lipoprotein-induced macrophage proliferation by inactivation of the small G protein-p38 MAPK pathway. J Biol Chem 2005;280:6627-33.
30. Jander S, Sitzer M, Wendt A, Schroeter M, Buchkremer M, Siebler M, et al. Expression of tissue factor in high-grade carotid artery stenosis: association with plaque destabilization. Stroke 2001;32:850-4.
31. Golledge J, Greenhalgh RM, Davies AH. The symptomatic carotid plaque. Stroke 2000;31:774-81.
32. Li C, Mollahan P, Baguneid MS, McMahon RF, Kumar P, Walker MG, et al. A comparative study of neovascularisation in atherosclerotic plaques using CD31, CD105 and TGF beta 1. Pathobiology 2006;73: 192-7.

33. Kawahara I, Kitagawa N, Tsutsumi K, Nagata I, Hayashi T, Koji T. The expression of vascular dendritic cells in human atherosclerotic carotid plaques. Hum Pathol 2007;38:1378-85.
34. Rothwell PM, Eliasziw M, Gutnikov SA, Warlow CP, Barnett HJ. Endarterectomy for symptomatic carotid stenosis in relation to clinical subgroups and timing of surgery. Lancet 2004;363:915-24.
35. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 2002;360:7-22.
36. Amarenco P, Labreuche J, Lavallee P, Touboul PJ. Statins in stroke prevention and carotid atherosclerosis: systematic review and up-to-date meta-analysis. Stroke 2004;35:2902-9.
37. Paciaroni M, Hennerici M, Agnelli G, Bogousslavsky J. Statins and stroke prevention. Cerebrovasc Dis 2007;24:170-82.

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